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Composition of molecular distillates of corn oil: isolation and identification of sterol esters*

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SUMMARY

Molecular distillates of corn oil consisting of the three most volatile 1.7 per cent cuts and the next most volatile 4.9 per cent cut obtained from 150 kg. of refined oil have been subjected to simple solvent and chromatographic fractionation. These fractions represented about 10 per cent of the original oil and some 50 per cent of its total unsaponifiable material. About 74 per cent of this unsaponifiable matter was sterol, of which 75 per cent was free, the rest combined—mainly as the long-chain fatty acid esters. The remainder of the analyzed unsaponifiable matter consisted of hydrocarbons (3.3 per cent), tocopherols (15.0 per cent), material more polar than free sterols (5.0 per cent), and various other uncharacterized fractions of differing polarity (2.7 per cent). The sterol ester fraction was made up largely of the linoleates, oleates, and palmitates of beta- and gamma-sitosterols and the triterpenoid alpha-sitosterols. Indications were obtained for the presence of tocopherol esters in corn oil. Among the glyceride esters the mono- and diglycerides of the major corn oil fatty acids were recognized representing, respectively, 0.05 and 0.52 per cent of the original oil. The refined corn oil was estimated to contain about 0.08 per cent acidic material of which more than 90 per cent appeared in the first most volatile 1.7 per cent cut. It contained mainly oleic and linoleic acids in about the same proportion as they occur in the corn oil.

Work performed in this laboratory (1, 2, 3) has demonstrated that some substance in the more volatile molecular distillates of corn oil, which are rich in unsaponifiable matter, accounts at least in part for the hypocholesterolemic effect of this foodstuff. Grande *et al.* (4) have reached a similar conclusion concerning the activity of the unsaponifiable matter of this oil. Of the compounds reported to occur in this fraction, alpha-tocopherol was shown to have no effect, whereas a preparation of beta-sitosterol, known to be grossly impure, did elicit a hypocholesterolemic response (1). This effect, first described by Peterson (5) in chicks and confirmed by Pollak (6) in man, has been demonstrated in many other laboratories (7), though a certain degree of variation has also been reported. This variation has been interpreted by some investigators (8, 9) as a result of a possible differential response on the part of the individual toward beta-sitosterol, a variation in the purity of the sterol preparation, or both. The possibility remains that plant sterols other than beta-sitosterol or indeed that some other type of compound associated with these substances may be primarily responsible for the hypocholesterolemic activity residing in the unsaponifiable matter of corn oil and its volatile molecular distillates.

In order to identify the agent or agents responsible for this hypocholesterolemic effect, extensive qualitative and quantitative analytical investigations of the unsaponifiable matter of corn oil enriched in the more volatile components have been undertaken. The availability of sufficient quantities of molecular distillates of this oil, provided through the courtesy of the Distillation Products Industries, of Rochester, New York, has greatly facilitated this study. In addition, the utilization of such distillates in these analyses has permitted an investigation of the naturally occurring but little-known plant sterol esters, thus yielding information impossible to obtain when working with the original oil or its alkaline hydrolysates. The following report describes methods found to be suitable for the fractionation of the above distillates and the isolation and preliminary characterization of several sterol and tocopherol esters as well as mono- and diglycerides, none of which have been hitherto reported to occur in corn oil.

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MATERIAL AND METHODS

The distillation constants and certain other characteristics of these distillates and the original oil are given in Table 1. All reagents and solvents employed were reagent grade chemicals and, unless otherwise indicated, were used without further purification.

TABLE 1. DISTILLATION CONSTANTS* AND CERTAIN OTHER CHARACTERISTICS OF CORN OIL DISTILLATES†

Fraction	Temperature	Pressure	Original Oil	Color	State at Room Temperature
			Original Distillation		
		μ.	*per cent*		
1st Cut	227°–230°C	13–15	10	orange	solids in liquid
2d Cut	228°–232°C	7–9	10	yellow	liquid
3d Cut	230°C	10	30	yellow	liquid
Residue	(not distilled)		50	yellow	liquid
			Redistillation of 1st Cut		
1 D	235°C	6	1.7	yellow-orange	solid
2 D	240°C	4	1.7	orange	solids in liquid
3 D	240°C	4	1.7	amber	liquid
4 D	245°C	4	4.9	yellow	liquid

* The distillation constants were recorded by Distillation Products Industries, Rochester, N. Y.

† The original oil (Mazola) had the following characteristics: specific gravity (25°C) 0.9190, smoke point (°F) 465, saponification equivalent 191, A. O. M. stability at 98°C (hr.) 19, iodine value 125, phospholipids 0.003%, tocopherols about 0.1%, unsaponifiable matter 1.55%, sitosterols 1%. The fatty acid composition was: myristic 0.2, palmitic 9.9, stearic 2.9, saturated above C_{18} 0.2, hexadecenoic 0.5, oleic 30.1, linoleic 56.2, and linolenic 0, all as per cent of total. (D. M. Rathmann, personal communication to J. M. R. Beveridge.)

Solvent Fractionation of the Distillates. The acidic and the neutral components were separated by extracting an ethereal solution of the distillate with 0.05 N KOH. Recoveries of the acidic fraction plus neutral fat were approximately 98 per cent. The bulk of the free sterol was removed from the 1 D distillate by crystallization from ethyl acetate and methanol.

Fractionation of Neutral Lipids. The method was essentially a combination of those described by Fillerup and Mead (10) and Barron and Hanahan (11). It was validated by performing test runs with a number of reference compounds, such as paraffin, squalene, octadecyl palmitate, alpha-tocopheryl palmitate, beta-sitosteryl palmitate, triolein, octadecanol, alpha-tocopherol, lanosterol, beta-sitosterol, ergosterol, diolein, mono-olein, and 1,18-octadecanediol.

Fractionation of Sterol Esters. The sterol ester mixtures isolated by adsorption chromatography from the various distillates were resolved into a number of bands using the reversed phase paper partition chromatography method described earlier (12). In order to obtain sufficient amounts of material for saponification and paper chromatographic analysis of the hydrolysis products, separations were performed on full sheets of double thickness Whatman No. 3 filter paper.

Fractionation of Sterols. The reversed phase partition chromatography system of Sulser and Hoegl (13) was modified. Either an aqueous 40 per cent butyric or 88 per cent acetic acid solution equilibrated with paraffin oil served as the mobile phase. Whatman No. 1 filter paper was used and the chromatograms developed by the ascending technique. The sterol mixtures were run as bands, as described for the sterol esters. The method was checked with purified beta- and gamma-sitosterol.

Fractionation of Fatty Acids. For the separation of the unsaturated fatty acids, the reversed phase partition chromatography method of Schlenk *et al.* (14) was used. Both saturated and unsaturated fatty acids were simultaneously separated and semiquantitatively estimated by the method of Inouye and Noda (15). The reliability of both techniques was verified with mixtures of known fatty acids.

Characterization of Glyceride and Sterol Esters. The distillates or any fraction thereof were saponified with a solution of 8 per cent (w/v) KOH in aqueous methanol (95 per cent), using a ratio of 3 g. of KOH to 1 g. of lipid. Samples requiring less than 25 ml. of alkali solution were diluted to 25 ml. with benzene. The unsaponifiable matter and the fatty acids were recovered in the usual way. The aqueous fraction was used for the assay of glycerol (16). The sterols were either converted into digitonides (17) and assayed for fast- and slow-acting components (18) or were chromatographed on paper. The fatty acids were separated and identified by chromatography.

The total and alpha-monoglycerides were estimated by the periodate oxidation according to the method of Martin (19).

The iodine numbers were determined by the micromethod of Yasuda (20). Optical rotations were taken on 2 per cent solutions in chloroform in a Hilger Model M 412 polarimeter. Ultraviolet spectra were recorded on absolute ethanol solutions on a Beckman Model DK 2 ratio recording spectrophotometer. Infrared spectra were determined on a Perkin-Elmer Infracord spectrophotometer equiped with sodium chloride optics. Qualitative colorimetric tests for ketosteroids,

TABLE 2. APPROXIMATE DISTRIBUTION OF UNSAPONIFIABLE MATTER DURING MOLECULAR DISTILLATION OF REFINED CORN OIL

Fraction	Original Oil	Total Unsaponifiable Matter		Sterols			Tocopherols	Hydrocarbons
	per cent	*per cent distillate*	*per cent original unsap.*	*per cent distillate*			*per cent distillate*	*per cent distillate*
				total	*free*	*bound*		
	Original Distillation*							
Original oil	100	1.65	100	1.1	—	—	0.12†	0.02†
1st cut	10	8.05†	48.7	5.95†	4.39†	1.56†	1.2†	0.25†
2d cut	10	2.5	15.1	2.2	—	—	—	—
3d cut	30	1.0	18.2	0.9	—	—	—	—
Residue	50	0.6	18.1	0.4	—	—	—	—
	Redistillation of 1st Cut							
1st cut	10	8.05†	48.7	5.95†	4.39†	1.56†	1.2†	0.25†
1 D	1.7	31	31.9	22.5	19.5	3	7.0	1.3
2 D	1.7	10	10.2	7.15	3.65	3.5	0.3	0.05
3 D	1.7	4.2	4.3	3.5	1.0	2.5	0.02	0.01
4 D	4.9	0.76	2.2	0.65	trace	0.65	0.01	0.0

* Wherever a dash is shown, the determination was not performed.
† Estimated from analyses on fractions 1 D–4 D.

vitamin A, tocopherols, amino acids, and carbohydrates were made by the Zimmermann, Carr-Price, Emmerie-Engel, ninhydrin, and Molisch reactions, respectively. Phosphorus was determined by the method of Beveridge and Johnson (21) and nitrogen by nesslerization.

RESULTS AND DISCUSSION

Preliminary Characterization of Distillates. From the physical appearance of the distillates (Table 1), it was obvious that the first and the second most volatile 1.7 per cent cuts contained large quantities of free sterol that had crystallized out. Digitonin precipitation of the free sterol before and after saponification of portions of the distillates and gravimetric determination of the amounts of digitonides obtained indicated that the distillates 1 D, 2 D, 3 D, and 4 D contained about 22.5, 7.15, 3.5, and 0.65 per cent of total sterol, respectively, of which 13.5, 49.0, more than 71, and more than 95 per cent, respectively, occurred in the combined form (Table 2). Since all the known corn oil sterols had been reported (22) to form readily insoluble digitonides, this method was considered a valid estimate of the free and esterified sterol content.

With the digitonides on hand, an attempt was made to estimate the content of the fast-acting sterols by determining the modified Liebermann-Burchard color reaction time curves. Though some of the digitonides partially decomposed during drying and did not permit a fully satisfactory examination, it was noted that in none of the distillates were there significant amounts of fast-acting sterols. The optical density time curves rose gradually, showing no peak or shoulder after a 90-second reaction time, reaching a maximum after about 30 minutes and superimposing to a considerable extent with the curve obtained for a purified beta-sitosterol sample when adjusted for differences in concentration.

Determination of the ultraviolet absorption spectrum of the 1 D distillate showed a maximum at 294 mμ. and a large shoulder at 285 mμ. with a minimum at about 267 mμ. The location of the maximum and minimum and the general shape of the curve (23, 24) indicated that the distillate contained large quantities of tocopherol. Since esterification had been known (24) to shift the absorption maximum to about 286 mμ., the shoulder observed at this wave length was taken as an indication of the possible presence of tocopherol esters. This belief was supported by the

TABLE 3. APPROXIMATE DISTRIBUTION OF SOME SAPONIFIABLE LIPID COMPONENTS DURING MOLECULAR DISTILLATION OF REFINED CORN OIL

Fraction	Original Oil	Free Fatty Acids	Monoglycerides	Diglycerides	Triglycerides	Unknown Material
	per cent	*per cent distillate*				
	Original Distillation*					
Original oil	100	0.08†	0.05†	0.52†	97†	0.37†
1st cut	10	0.84†	0.49†	5.22†	80.66†	3.7†
2d cut	10	—	—	—	95	—
3d cut	30	—	—	—	98	—
Residue	50	—	—	—	99	—
	Redistillation of 1st Cut					
1st cut	10	0.84†	0.49†	5.22†	80.66†	3.7†
1 D	1.7	4.7	2.5	16.03	35	7.5
2 D	1.7	0.2	0.4	10.70	70	5.30
3 D	1.7	0.02	0.01	0.51	87	5.76
4 D	4.9	0.00	0.00	0.01	98	1.23

* Wherever a dash is shown, the determination was not performed.
† Estimated from analyses on fractions 1 D–4 D.

observation that an alkaline hydrolysis destroyed the shoulder with a concomitant increase in the maximum and decrease in the minimum absorption. The 2 D, 3 D, and 4 D distillates showed two maxima each at about 270 mμ. and 280 mμ., as did the original oil, superficially resembling the absorption curve for a delta-5,7-diene sterol. On saponification, however, none of this ultraviolet absorbing material appeared in the unsaponifiable matter, and consequently could not be attributed to ergosterol as was done by Heilbron *et al.* (25). The report of these workers claiming the presence of ergosterol in corn oil on the basis of ultraviolet light absorption has not been confirmed by an actual isolation of this sterol from corn oil or its unsaponifiable matter. The apparent absence of any significant amounts of fast-acting sterol from these distillates makes it unlikely that ergosterol is present in corn oil. Using an extinction coefficient $E^{1\%}_{1\ cm.}$ of 90 for a mixture of the corn oil tocopherols in ethanol solution at 294 mμ., the total tocopherol concentration in the pooled distillates was estimated at about 1 per cent. If all the tocopherol had distilled over into the first 10 per cent of the original distillation, this would represent a concentration of about 100 mg. of tocopherol per 100 g. of the refined oil, a figure well within the range of values recorded in the literature (26).

Determination of the neutral equivalent of the distillates by titration of suitable oil samples in chloroform with methanolic KOH and assuming an average molecular weight of 280 for the fatty acids of corn oil indicated that the 1 D distillate contained about 4 per cent of free fatty acids. Only traces of acidic material were detected in the other distillates (Table 3).

In addition, the distillates were analyzed for nitrogen and phosphorus by methods capable of detecting one part per million. While no phosphorus could be detected in any of the distillates, there were about 5 parts per million of nitrogen detected in the most volatile fraction, which, however, could not be associated with any of the fractions recovered from the silicic acid column, when spot checks on the eluted lipids were made. The fractions gave negative reactions for carbohydrates in the Molisch test.

Simple Solvent Fractionation of Distillate 1 D. During the initial characterization of the various distillates it was noticed that the 1 D distillate contained significant amounts of acidic material. Removal of this material by extraction with a mild alkali provided an easy access to it and considerably simplified the subsequent adsorption chromatographic fractionation. The small amounts of free fatty acids left in the other

distillates overlapped with the triglycerides when chromatographed on the silicic acid. The amount of the acidic material extracted from 1 D amounted to about 5 per cent of the distillate and was in fairly good agreement with the estimate of the free fatty acid content of 1 D obtained from the neutral equivalent determination.

The adsorption chromatographic fractionation of the neutral lipid of distillate 1 D was further facilitated by a preliminary removal of the bulk of the free sterol. Ethyl acetate-methanol recrystallization of 1 D removed approximately 17 per cent of crystalline material, part of which (0.5 per cent of 1 D) was a solid hydrocarbon (m.p. 50°C). The rest of the crystalline material consisted of a mixture of free sterols (m.p. 136°-138°C, α_D — 31°). Further solvent fractionation of the neutral 1 D lipid into material soluble and insoluble in absolute methanol, partition between methanol and ligroin, and low temperature crystallization from acetone were tried. These techniques produced a great variety of fractions but failed to yield any significant separation or noticeable simplification of the subsequent chromatography.

Adsorption Chromatography of Unsaponifiable Matter. Silicic acid chromatography permitted the fractionation of the unsaponifiable matter from the various distillates into several main groups of compounds. The elution pattern obtained with the 1 D distillate is represented in Figure 1; the approximate composition of the fractions is shown in Table 4. Essentially the same elution patterns were obtained with the unsaponifiable fractions of other distillates, except for diminished amounts of the more volatile substances. The results of these analyses of the various unsaponifiable fractions from the four distillates are summarized in Table 2. It may be noted that the figures obtained for the total sterol are in good agreement with those arrived at during the preliminary characterization of the various distillates by gravimetric digitonide determinations. The figures for total tocopherol are somewhat higher, but this was to be expected, as the spectrophotometric measurements made on the unsaponified distillates failed to correct for the lower extinction of the tocopherol ester at the wave length of analysis.

About 100 mg. of an unsaturated hydrocarbon, at least part of which was squalene, as indicated by the formation of a hydrochloride of correct melting point and approximately correct iodine number, was isolated from this unsaponifiable fraction. It corresponds to the amount present in about 31 g. of distillate 1 D. Assuming that all the unsaturated hydrocarbon was

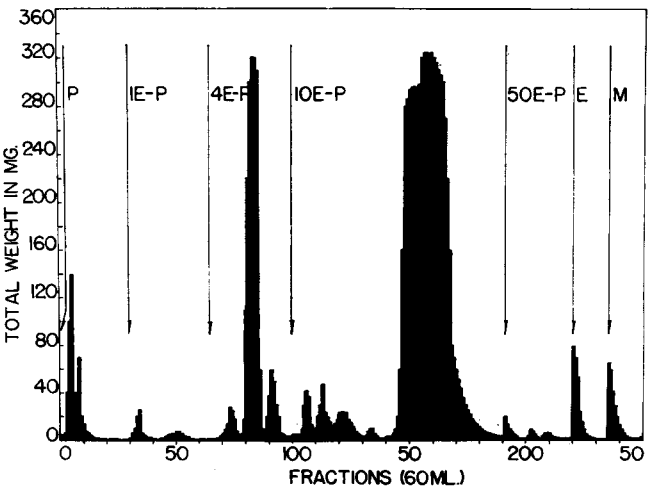

FIG. 1. Chromatography on silicic acid of the unsaponifiable matter from corn oil distillate 1 D. Solvents were petroleum ether (P), diethyl ether (E), and methanol (M). The composition of the various fractions is recorded in Table 4.

squalene and that all squalene distilled over into the 1 D fraction, this amount would fall within the range of squalene concentrations reported by Fitelson (27) for corn and other seed oils. However, the absorption spectrum of this unsaturated hydrocarbon indicated that there was also some carotene present. It appears that the quantities of both squalene and carotene isolated represented only part of these substances originally present in the corn oil, as it is known that at least carotene, because of its high unsaturation, distills irregularly (28) under the molecular distillation conditions and is polymerized (29) and peroxidized in the process. Further degradation of the more unsaturated components of these distillates took place during the analytical manipulation (fraction VIII, Table 4).

Adsorption Chromatography of Neutral Lipids. The preliminary removal of the bulk of the free sterol and all the acidic material was both convenient and desirable for a successful adsorption chromatographic fractionation of the residue. Though with carefully adjusted solvent polarity it has been possible to separate free fatty acids from triglycerides (30) and free sterols, usually the acids are eluted together with the triglycerides (11). The sterol removal rendered the residue much more soluble in the solvents of low polarity, making the initial adsorption of the material on the adsorbent column possible from reasonable quantities of petroleum. It also permitted a rather clean-cut resolution of the free sterol and the diglyceride peaks. The nature of the fractionation pattern of the lipid remaining in 1 D after the removal of the free fatty acids and most of the free sterols is graphically depicted in Figure 2. The solvents and their vol-

TABLE 4. Composition of Fractions Obtained on Silicic Acid Chromatography of the Unsaponifiable Matter from Corn Oil Distillate 1 D*

Fractions in Order of Elution	Eluting Solvent	Volume of Eluting Solvent	Components	Fraction Weight	Original Material	Remarks
		ml.		*mg.*	*per cent*	
I	Petroleum ether	1800	Saturated hydrocarbons	300	3.0	Waxy solid, m.p. 30°–50°C, iodine number 0.7
			Squalene and carotene	100	1.0	Yellow oil, λ_{max} 440 mμ., HCl, m.p. 122°C, iodine number 359
II	1% ethyl ether in petroleum ether	2000	Ketones	50	0.5	White semisolid, slightly Zimmermann positive, iodine number 55
III	4% ethyl ether in petroleum ether	2000	Tocopherols, alcohols, C_4 Me sterols	1960	20.0	Yellow oil, λ_{max} 294 mμ., λ_{min} 267 mμ., Emmerie-Engel positive
IV	10% ethyl ether in petroleum ether	2000	Sterols	450	4.5	Cryst. solid, L-B positive, m.p. 126°–132°C, α_D −10 to −24°
V	10% ethyl ether in petroleum ether	3000	Sterols	6500	66.0	Cryst. solid, L-B positive, m.p. 136°–138°C, α_D −30°
VI	50% ethyl ether in petroleum ether	1800	Sterols	35	0.3	Cryst. solid, L-B positive, m.p. 138°–140°C, α_D −20°
VII	Absolute ethyl ether	1000	Unidentified alcohols	300	3.0	Amber-colored oil, L-B negative, α_D 0.6°
VIII	10% methanol in ethyl ether	1000	Unknown	300	3.0	Dark red glass, probably peroxidized squalene and carotene degradation products, L-B negative, α_D 0°

* 9.85 g. of lipid was applied to 600 g. of silicic acid. The total recovery was 9.99 g. (101%). See Figure 1 for the elution pattern.

umes used together with the composition of the major fractions and certain remarks are recorded in Table 5. Similar elution patterns were obtained on silicic acid chromatography of the other distillates, except that there was less of the more volatile material present, with a consequent increase in the proportion of the triglyceride. Table 3 summarizes the data obtained for the major saponifiable lipid components present in the various distillates.

The fractions obtained by this means, although apparently clearly separated, were found to be contaminated with preceding or succeeding fractions, or both. A similar observation has been reported by Lovern (31), who, working with the lipids of haddock flesh, obtained quite sharp peaks on silicic acid chromatography, but subsequently found that these components did not necessarily represent pure fractions. For further study, such as the sterol and sterol ester separations described below, or for a quantitative assay, the fractions had to be rechromatographed, preferably with the same solvent sequence as that used in the original experiment.

In view of the results presented, it appears that most of the sterol in corn oil exists in the free state. However, the distillates analyzed contain only a part of the total free and combined sterol present in the original oil, since significant amounts of the unsaponifiable material, much of which is sterol, have been left behind in the second and third most volatile cuts and in the residue from the original distillation (Table 2). Because of the greater volatility of the free sterol (32), the unsaponifiable matter (51.3 per cent of original unsaponifiable matter) left behind in the bulk of the oil during the original distillation, is likely to contain a much greater proportion of the combined sterol form. The elimination of only about 50 per cent of the total unsaponifiable matter of corn oil into the first most volatile 10 per cent fraction during the

original distillation should be contrasted with an elimination of about 95 per cent of the total unsaponifiable material of soybean oil into the corresponding fraction during a similar distillation (33). Assuming that sterols make up most of the unsaponifiable matter in both oils, and knowing that the sterol esters are considerably less volatile than the free sterols, it would appear that a large sterol ester concentration, such as observed for the corn oil, might not be the property of all plant oils.

Estimation of alpha-mono- and the total monoglyceride content before and after perchloric acid isomerization (19) indicated that practically all (97 per cent) of the monoglyceride was present as the 1-monoglyceride. An estimation of the proportion of the major fatty acids, e.g., palmitic, oleic, and linoleic, present in the various glycerides and the original oil by paper chromatography (15), indicated an approximate ratio of 10, 40, and 50, respectively (cf. also 34). An approximately similar ratio for these fatty acids was also observed for the sterol ester fraction, but the presence of other fatty acids was indicated.

Naturally occurring plant sterol esters have been isolated only rarely (35), usually by accident, since the small amounts present in common lipid extracts may be lost in any large-scale systematic analyses. This is easily understood since techniques apparently suitable for obtaining lipid preparations enriched with sterol esters, such as molecular distillation (28, 36) and preferential enzymatic destruction of the triglyceride (37, 38) and their effective fractionation by adsorption chromatography (10, 11, 30), have been exploited only recently. The recent publication of the paper partition chromatographic methods for the separation of beta- and gamma-sitosterol mixtures (13) and the long-chain fatty acid esters of sterols (12, 39) has now permitted an examination of this class of compounds.

TABLE 5. Composition of Fractions Obtained on Silicic Acid Chromatography of the Neutral Lipids (Low in Free Sterols) from Corn Oil Distillate 1 D*

Fractions in Order of Elution	Eluting Solvent	Volume of Eluting Solvent	Components	Fraction Weight	Original Material	Remarks
		ml.		*mg.*	*per cent*	
I	Petroleum ether	1800	Hydrocarbons	210	2.0	Waxy solid, m.p. 30°–50°C, plus a yellow oil, iodine number 387
II	1% ethyl ether in petroleum ether	2500	Aliphatic alcohol, tocopherol esters and sterol esters	1380	13.0	Light yellow oil, L-B positive, λ_{max} 285 mμ., λ_{min} 260 mμ., F.A. 41%, sterol 45%, tocopherol 11%, unacc. 3%
III	4% ethyl ether in petroleum ether	2600	Triglycerides and tocopherols, alcohols, C_4 Me sterols	3720	35.0	Light yellow oil, λ_{max} 294 mμ., λ_{min} 267 mμ., 20% tocopherol, fatty acid/glycerol molar ratio 3.2
IV	10% ethyl ether in petroleum ether	5400	Residual sterols and tocopherols	2660	25.0	Solid, L-B positive, 1% tocopherol; sterol recryst. m.p. 132°C, α_D −28°
V	25% ethyl ether in petroleum ether	3600	Diglycerides	1700	16.0	Colorless oil, slightly L-B positive, fatty acid/glycerol molar ratio 2
VI	50% ethyl ether in petroleum ether	1800	Unknown alcohols	75	0.8	White oil, fatty acid/nonlipid alcohol molar ratio 1.5
VII	Absolute ethyl ether	1800	Monoglycerides	320	3.0	Colorless oil, fatty acid/glycerol molar ratio 1.2
VIII	10% methanol in ethyl ether	2500	Unknown	530	5.1	Dark red solid, probably peroxidized squalene and carotene degradation products

* 10.63 g. of lipid was applied to 600 g. of silicic acid. The total recovery was 10.59 g. (100%). See Figure 2 for the elution pattern.

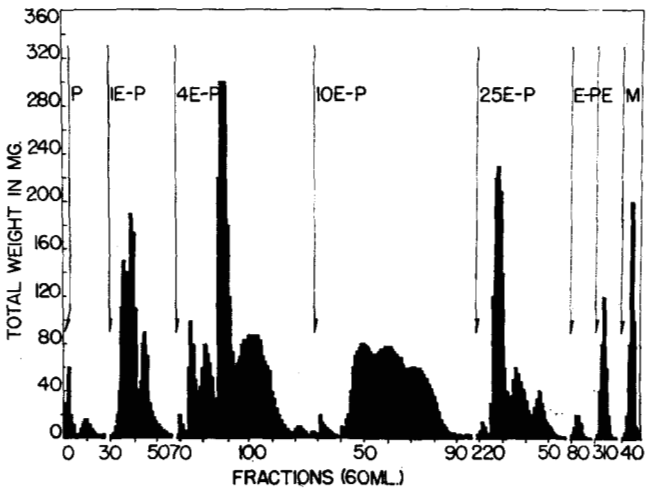

FIG. 2. Chromatography on silicic acid of the neutral lipids (low in free sterols) from corn oil distillate 1 D. Solvents as in Figure 1. E-P indicates 50 per cent diethyl ether in petroleum ether. The composition of the various fractions is recorded in Table 5.

Partition Chromatographic Fractionation of Components Isolated from the Distillates Following Adsorption Chromatography. Because of the nature of the project (see first two paragraphs) of which this study was a part, interest was focused primarily on the free sterols and sterol esters. As a result of certain observations during the preliminary paper chromatography of lipid mixtures rich in sterol esters, it was noticed that the application of reversed phase paper partition chromatography would provide a quick indication of the nature of the sterol esters present. Several solvent systems were developed which permitted a satisfactory resolution of all the even-numbered fatty acid esters of a given plant sterol (12). Difficulties, however, were observed when several esterified sterols were present. Thus, in the case of the corn oil sterol esters, at least three different sterols provided the alcohol parts of the esters. When such an ester mixture was chromatographed in these systems, several discrete bands were obtained (Fig. 3). In order to identify these bands, it was necessary to collect at least sufficient amounts of material for saponification and subsequent paper-chromatographic analyses of the hydrolysis products. This was accomplished by scaling up the above reversed phase paper partition chromatography method for preparative purposes. Using this method, as much as 50 mg. of the mixed sterol esters were eventually separated into the different bands (Fig. 3). The bands were eluted, the lipids saponified, and the fatty acids and the unsaponifiable matter recovered separately. The acids were chromatographed in the reversed phase systems described by Schlenk *et al.* (14) and Inouye and Noda (15). The latter method permitted the resolution of the "inseparable" pairs of the fatty acids and a semiquantitative estimation. The unsaponifiable parts of the esters were chromatographed, using a modification of the method described by Sulser and Hoegl (13). It permitted the separation of beta- and gamma-sitosterol, the alpha-sitosterol(s), saturated sterols, and tocopherols present in the unsaponifiable matter from the mixed sterol esters (Fig. 4).

As a result of these analyses, it was possible to demonstrate that the separation of the esters (Fig. 3) had taken place primarily on the basis of the fatty acid moieties. Thus the beta- and gamma-sitosteryl oleates had traveled together (band I) but had overlapped to some extent with the palmitic acid derivatives. The next fastest moving band, H, was that of beta- and gamma-sitosteryl linoleates. Figure 5 shows the sterol composition of the mixed sitosteryl linoleate fraction from band H. An identical chromatogram was obtained for the sterols present in the mixed oleate fraction (band I). The next band, G, in Figure 3, was due to the oleate of a sterol moving faster than either beta- or gamma-sitosterol, assumed to be alpha-sitosterol, and minor amounts of as yet unidentified fatty acid esters of beta- and gamma-sitosterols. This band also contained some tocopherol ester which, on the basis of its R_f value, was believed to be the oleate. The composition of the sterols of band G is shown in Figure 6. The fastest moving ester band, F, in Figure 3 was thought to be due to the linoleate of the alpha-sitosterol and another tocopherol ester, possibly the linoleate. The fatty acid part of band F also contained at least one additional unsaturated fatty acid other than linolenic, which moved faster than linoleic.

The sterol bands were identified on the basis of their color reactions and the R_f values. The reliability of the latter criterion was checked by performing the chromatographic separations of the unknowns with and without the addition of known standards of the beta- and gamma-sitosterols. In the absence of any alpha-sitosterol standards, the identification of the alpha-sitosterol band is only tentative. Anderson and Shriner (22) suggested that alpha-sitosterol occurs in corn oil in fair amounts and this suggestion has been accepted by us as a working hypothesis. That this band was not due to stigmasterol was demonstrated by the observation that the latter formed a distinct and somewhat faster moving band than the unknown compound. The triterpene dienol structure recently suggested for alpha-sitosterols by Mazur *et al.* (40)

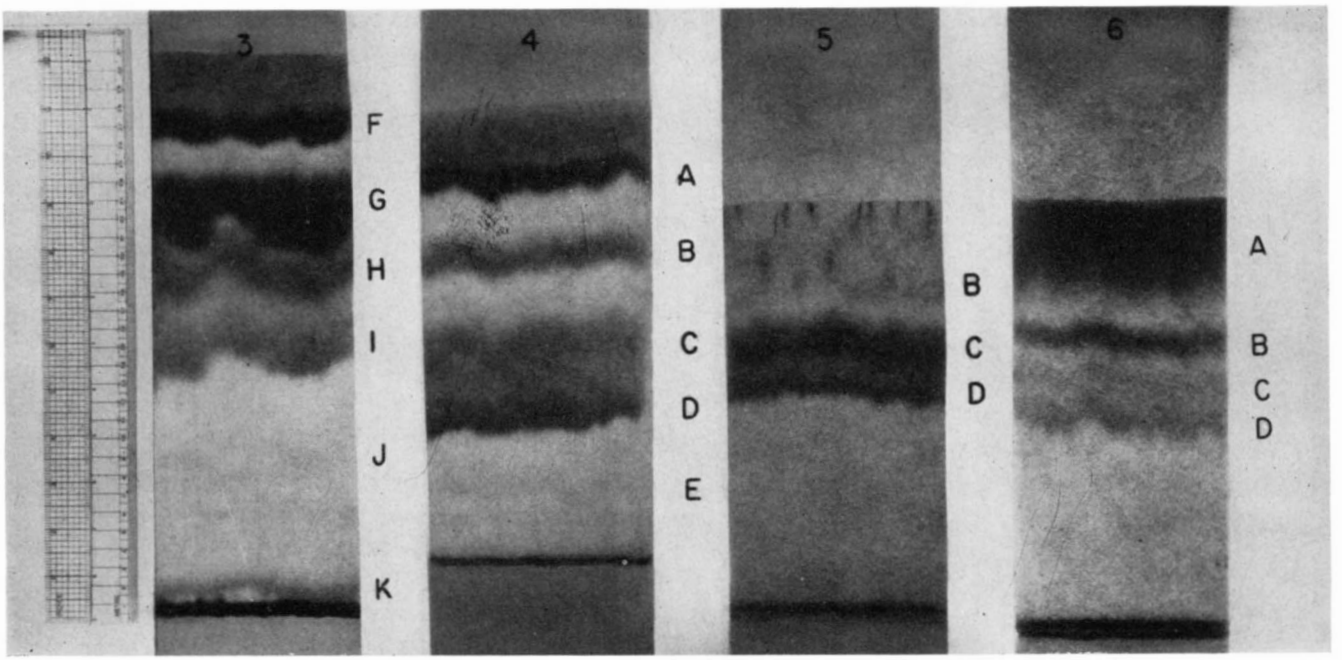

FIG. 3. Chromatography of corn oil tocopherol and sterol esters. System: 90% propionic acid/paraffin oil on paraffinized Whatman No. 1 filter paper. Chromatogram stained with iodine vapor. F = tocopherol and alpha-sitosteryl linoleate (tentative), G = alpha-sitosteryl oleate, H = beta- and gamma-sitosteryl linoleates, I = beta- and gamma-sitosteryl oleates and palmitates, J = unknown, K =unidentified esters of beta- and gamma-sitosterols.

FIG. 4. Chromatography of free tocopherols and sterols from the corn oil sterol ester hydrolysate. System: 88% acetic acid/paraffin oil on paraffinized Whatman No. 1 filter paper. Chromatogram stained with iodine vapor. A = tocopherols, B = alpha-sitosterol(s), C = gamma-sitosterol, D = beta-sitosterol, E = saturated sterol (?).

FIG. 5. Chromatography of free sterols from the pooled steryl linoleate (band H, Fig. 3) hydrolysate. System and code as in Figure 4.

FIG. 6. Chromatography of free sterols from the pooled alpha-sitosteryl oleate (band G, Fig. 3) hydrolysate. System and code as in Figure 4.

might account for the slightly lower polarity of this compound in comparison to stigmasterol in the reversed phase partition system. The tocopherol bands were identified after elution and determination of the ultraviolet spectra. On paper, these bands first turned yellow, then red, after standing in the air for periods of 24 hours or more.

A similar paper chromatographic examination of the sterol present in the free form in the corn oil distillates showed the predominance of the beta- and gamma-sitosterols. The sterols obtained on crystallization of 1 D gave only two readily detectable sterol bands, one for beta- the other for gamma-sitosterol. The free sterols obtained on adsorption chromatography of the neutral lipids from the various distillates on silicic acid contained, in addition to these two sterols, also significant quantities of the alpha-sitosterol. Though the sterol and tocopherol bands obtained on reversed phase paper partition chromatography appeared to be fairly sharp and distinct (Figs. 4, 5, and 6), it was felt that they could have hidden other minor components. It has been the experience of the authors with the reversed phase partition systems (12) that only suitably proportioned solute concentrations may be satisfactorily partitioned. A large excess of one component readily masks the minor component unless they differ greatly in their polarities. Therefore attempts are currently being made to effect an enrichment of the minor sterol and sterol ester components so that more satisfactory chromatographic examination may be performed.

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